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LACTOPEROXIDASE ACTIVITY IN GUINEA-PIG MILK AND SALIVA: CORRELATION IN MILK OF LACTOPEROXIDASE WITH BACTERICIDAL ACTIVITY AGAINST *ESCHERICHIA COLI*

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Summary.—The lactoperoxidase (LPO) activity in guinea-pig milk and saliva has been investigated in sows suckling normal young, and young orally infected with *Escherichia coli*. There was a 5-fold increase in activity in milk during the 3–4 weeks of lactation; infection of the young did not alter this. There was no comparable increase in lactoperoxidase activity of saliva during this same period, either in the infected or non-infected group.

The antibacterial activity of milk from sows suckling normal young increased with the lactoperoxidase, and this bactericidal activity could be reversed by LPO inhibitors such as penicillamine and cysteine but not by addition of sufficient iron to saturate the lactoferrin. In milk from sows suckling infected young, bacteriostatic activity occurring in samples from about 14 days after infection needed iron or both iron and penicillamine (or cysteine) for reversal, indicating that both the antibody–lactoferrin system and the LPO system may be involved in the infected state.

IT HAS BEEN KNOWN for some time that milk has antibacterial properties and several such systems have been described (Hanson and Winberg, 1972). Hanssen (1924) described an antibacterial property which correlated well with the presence of oxidizing enzymes in the milk; Jago and Morrison (1962) showed that a purified preparation of lactoperoxidase (LPO) from milk would inhibit the growth of *Streptococcus cremoris in vitro* and that the hydrogen peroxide produced by the organism was essential for the inhibition. Reiter *et al.* (1963) demonstrated that thiocyanate ions (SCN^-) were also necessary for the bactericidal activity and Klebanoff and Luebke (1965) suggested the bactericidal activity of saliva was due to the same enzyme. Further work (Klebanoff, Clem and Luebke, 1966; Hamon and Klebanoff, 1973) has confirmed these findings, and Bjorck *et al.* (1975) have shown the system to be effective against Gram-negative organisms including *Escherichia coli*. Thomas and Aune (1978) have also investigated the susceptibility of *Esch. coli* to this system and have shown that alteration of the cell envelope by agents such as lysozyme greatly enhances the bactericidal action of the lactoperoxidase system.

Bullen, Rogers and Leigh (1972) have described an *in-vitro* bacteriostatic activity in human milk involving lactoferrin and antibody, and this activity is reversed by addition of sufficient iron to saturate the lactoferrin. Their experiments in guinea-pigs suggested this system may be important *in vivo* in protecting suckling young against an oral challenge with *Esch. coli*.

In a previous paper (Stephens and Dolby, 1978) we described the development of an *in-vitro* bacteriostatic activity in guinea-pig milk in response to oral infection of the young which was reversed by iron. Our initial assays of lactoperoxidase in guinea-pig milk showed very high activity when compared with bovine milk, and this paper describes further investigations into the amounts of LPO found in milk and saliva and attempts to correlate it with the bactericidal and bacteriostatic properties of milk found in the non-infected

state and in response to oral infection of the young.

MATERIALS AND METHODS

Animals.—Hartley guinea-pigs were mated from 3 months of age and allowed to litter naturally and suckle their own young.

Milk and saliva samples.—Sows were milked from 12 h *post partum*; the young were removed from the mother for 4 h before milking during the first 10 days; after this, overnight separation was necessary to obtain adequate milk supply. The milk was expressed manually, tested within 1 h and then stored at −28°.

After collection of each milk sample, saliva production was stimulated by exposing the sows to ether vapour for 1–2 min. The saliva was collected using a plastic pipette and samples were tested within 2 h of collection.

Infection of guinea-pigs.—The young were infected orally at 2–4 days of age with milk-resistant strains of *Esch. coli* 11(2)B1-1 and 17(2)B2-2 by the method described previously (Stephens and Dolby, 1978).

Bacteriostatic/bactericidal test.—Milk samples were incubated in 0·08ml volumes with 0·02 ml saline containing approximately 5×10^3 viable *Esch. coli* organisms. Both the milk-sensitive strain V21-1 and the milk-resistant strain 11(2)B1-1 were used for these tests. Results were expressed as the $\log_{10}$ increase or decrease in inoculum after 3 h at 37°. Milks were considered to have bactericidal activity if there was a decrease in inoculum, and bacteriostatic activity if there was less than 1 $\log_{10}$ increase after the 3 h of the test. The following compounds were added in 0·02ml volumes to give the indicated final concentrations: L-cysteine hydrochloride (Hopkins and Williams, Essex) 10mM; D-penicillamine (Lilly Research Centre Ltd, Surrey) 10mM; ferric ammonium citrate (B.D.H. Chemicals Ltd, Dorset) 20–20,000 μg/ml.

Lactoperoxidase assay.—Activities were estimated using the conditions described by Schindler, Childs and Bardsley (1976) for the substrate ABTS (Boehringer Ltd, London, W5). Samples were assayed immediately, although storage at −20° or −28° was not associated with any reduction in activity. Pasteurization of 5 samples at 63° for 30 min was only associated with a mean reduction in activity of 11%. For the assay of untreated milk samples, aliquots of 10 μl of guinea-pig milk, 30 μl of bovine milk and 50 μl of human milk were used in a final assay volume of 3 ml. Buffered ABTS substrate and milk were preincubated for at least 5 min at 25° in the spectrophotometer to ensure the formation of an even suspension with a stable absorbance. The reaction was then started by the addition of hydrogen peroxide. Activities in 5μl aliquots of saliva were estimated by the same method. Activities were expressed as milliunits per ml of milk or per mg salivary protein as determined by the method of Lowry *et al.* (1951) using a bovine serum albumin standard. One unit of activity was the amount of enzyme catalysing the oxidation of 1 μmol of ABTS per min at 25°.

The chemical nature, redox and spectroscopic properties of the system ABTS, H_2O_2 and horseradish peroxidase have been described (Childs and Bardsley, 1975). The complex

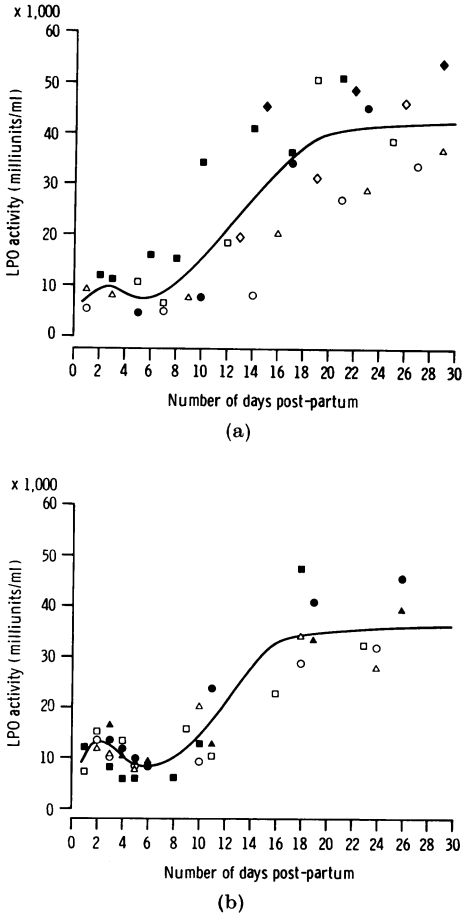

FIG. 1.—Lactoperoxidase activity of milks from sows suckling normal (a) and infected (b) young. Guinea-pigs are identified by individual numbers with litter numbers in parentheses. (a) (■) 93(1), (△) 95(1), (○) 88(2), (□) 104(1), (●) 101(1), (◇) 96(1), (◆) 94(1). (b) (■) 90(1), (△) 91(1), (○) 102(1), (□) 86(2), (●) 93(2), (▲) 74(3) Young were infected 2–4 days *post partum*. Lines are drawn through means calculated at 2–5-day intervals.

TABLE I.—*Lactoperoxidase Activity in Human, Bovine and Guinea-pig Milks and Guinea-pig Saliva*

Specimen	No. samples	Time *post partum*	Activity, milliunits/ml Range	Average
Human milk	9	1–69 days	26–519	226
Bovine milk	11	3–30 weeks	738–3,889	1,422
Guinea-pig milk	73	1–30 days	4,569–54,500	22,000
Guinea-pig saliva	52	1–30 days	316–3,778*	1,354

* Activity expressed as milliunits/mg protein.

TABLE II.—*Inhibition of Lactoperoxidase Activity of Guinea-pig Milk by Penicillamine*

Guinea-pig	Days *post partum*	Days post infection	Activity in milliunits/ml Milk	Milk + Penicillamine 10mM	% Inhibition
90 (1)*	8	5	6,023	4,257	29
74 (3)	3	0	12,174	9,628	21
74 (3)	5	1	7,826	5,022	36
74 (3)	19	15	23,639	17,185	27
96 (1)	19	0	31,056	23,806	23

* Litter number in parentheses.

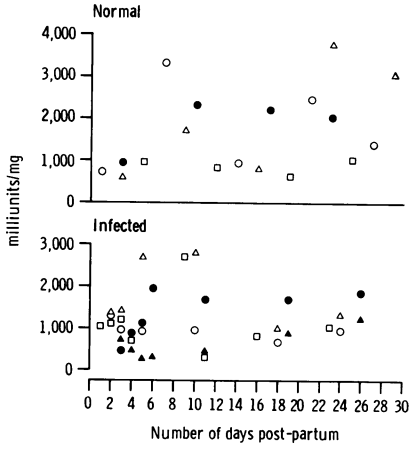

FIG. 2.—Lactoperoxidase activity in saliva from sows suckling normal and infected young. For symbols see Fig. 1. Activities are expressed per mg of salivary protein.

kinetics of lactoperoxidase appear to be similar to those of horseradish peroxidase (Schindler and Bardsley, 1975).

RESULTS

Lactoperoxidase activity

The activities found in milks from sows with normal young (Fig. 1a) and those suckling young orally infected with *Esch. coli* (Fig. 1b) were similar. Activities ranged from 5,000 to 15,000 milliunits/ml during the first week of lactation and increased to 30,000–50,000 milliunits/ml by the third week. These activities are high compared with human and bovine milks tested (Table I), suggesting this enzyme may play a more important role in guinea-pig milks than other milks.

LPO activity is inhibited by agents such as penicillamine, and assays on milks incubated 1 h at 37° with 10mM penicillamine showed an average 28% inhibition of activity (Table II).

LPO activities in the saliva of sows ranged from 316 to 3778 milliunits/mg protein (Table I) ,and no difference was found between the infected and non-infected groups (Fig. 2). The activity did not increase markedly during the *post-partum* period as it did in milk.

Bacteriostatic/bactericidal activity

Figs. 3a and b show the antibacterial activity of milk from 7 sows with normal young against the milk sensitive (V21-1) and milk-resistant (11(2)B1-1) strains of *Esch. coli*. The milk from 5 sows taken

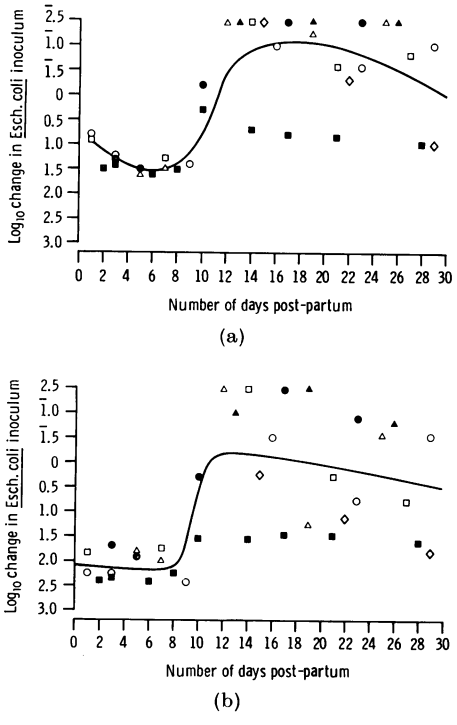

FIG. 3.—Bactericidal activity of milks from sows suckling normal young against a milk-sensitive (a) and milk-resistant (b) strain of *Esch. coli*. (■) 93(1), (○) 95(1), (□) 88(2), (△) 104(1), (●) 101(1), (▲) 96(1). (◇) 94(1). Lines are drawn through means calculated at 2–5-day intervals.

during the first week of lactation did not inhibit the growth of either strain. By the 16th day *post partum* milk samples from all sows with normal young were bacteriostatic against the milk-sensitive strain V21-1, and 5 out of 7 sows were secreting milk bactericidal for both strains. This activity was reversed by penicillamine and cysteine but not by iron, even in concentrations up to 20,000 μg/ml. Table III shows examples of serial milkings from 3 sows with normal young, 95 (1), 88 (2) and 104 (1), against milk-sensitive strain V21-1. The bactericidal activity generally increases with lactoperoxidase activity, but in some milks *e.g.* 88 (2), 14 days *post partum*, the bactericidal activity is high but the lactoperoxidase is still relatively low.

Table IV shows examples of milks from 3 of a series of 6 sows suckling infected young. Unfortunately in this group milks were frequently bacteriostatic against the milk-sensitive strain before infection. However this activity was always reversed by iron and was attributed to lactoferrin. Immediately following infection, iron alone was frequently insufficient to reverse the activity entirely and both iron and penicillamine or cysteine were necessary. Milks collected later in lactation varied in their

TABLE III.—*Lactoperoxidase and Bactericidal Activity of Milk from Sows Suckling Normal Young*

Guinea-pig	Days *post partum*	LPO activity in milliunits/ml	Bactericidal activity* with additives‡				
			—	Fe	Pen	Cyst	Fe+ Pen or Cyst
95 (1)	3	8,600	23	65	—†	—	—
	16	20,800	0·1	0·1	12	—	0·6
	23	29,250	0·4	0·1	7	—	—
	29	37,000	<0·1	—	21	—	—
88 (2)	7	5,000	25	—	—	—	—
	14	8,300	<0·1	<0·1	27	—	—
	21	27,000	0·3	—	27	—	—
	27	34,000	0·2	<0·1	33	—	—
104 (1)	7	6,800	4	0·8	50	—	—
	12	18,800	<0·1	<0·1	—	15	<0·1
	19	51,000	<0·1	<0·1	—	3	42
	25	39,500	<0·1	—	—	3	53

* Expressed as the -fold increase or decrease in inoculum of *Esch. coli* strain V21-1 after 3 h at 37°C.
† Not tested.
‡ Fe—ferric ammonium citrate 20–20,000 μg/ml, Pen—penicillamine 10mM, Cyst—cysteine 10mM.

TABLE IV.—*Lactoperoxidase and Bactericidal Activity of Milk from Sows Suckling Infected Young*

Guinea-pig	Days *post partum*	Days post infection	LPO activity in milliunits/ml	Bactericidal activity* with additives‡				
				—	Fe	Pen	Cyst	Fe+ Pen or Cyst
74 (3)	4	0	10,700	0·2	38	0·2	0·2	86
	5	1	9,200	<0·1	2	0·2	0·1	32
	11	7	12,800	2	2	15	2	59
	26	22	39,000	6	41	13	8	58
102 (1)	2	0	13,800	10	—†	—	—	—
	3	1	10,600	0·2	16	—	0·1	41
	10	8	9,600	7	37	—	11	120
	18	16	29,000	<0·1	0·3	—	7	62
90 (1)	1	0	12,060	0·8	35	23	—	56
	8	5	6,023	5	98	22	—	—
	18	15	47,500	14	37	26	—	—

For legend see Table III.

activity, but bactericidal milks were found less frequently than in the non-infected group, and bacteriostatic milks required both iron and penicillamine (or cysteine) for maximum reversal of activity, suggesting involvement of the lactoferrin–antibody system in the antibacterial activity as well as lactoperoxidase.

DISCUSSION

Two antibacterial systems have been investigated in guinea-pig milk, the lactoferrin/antibody system described by Bullen *et al.* (1972), which is reversed by saturation of the lactoferrin with iron, and the LPO-SCN^--H_2O_2 system described by Reiter *et al.* (1963), which is reversed by addition of reducing agents such as cysteine (Klebanoff, 1967) or inhibited by agents such as penicillamine (Renz, Nicol and Harkness, 1972). Previous work (Stephens and Dolby, 1978) has shown the lactoferrin/antibody system to be responsible for *in-vitro* bacteriostatic activity in guinea-pig milk following oral infection of the young with *Esch. coli*.

These further studies on guinea-pig milk from sows with normal young show very high lactoperoxidase activities, the average being 100 times greater than that found in human milk and almost 20 times greater than bovine milk tested. Serial milkings taken throughout the lactation period show a small rise in activity immediately *post partum* followed by a fall, with a further 5-fold rise after Day 8 from 8,000 milliunits/ml to 40,000 milliunits/ml by about Day 18.

The increases in LPO activities are similar to the increases in oestrogen production which are associated with the oestrous cycle. A small rise in oestrogen excretion is associated with the *post partum* oestrus of the guinea-pig (Lea, Bessesen and Stoa, 1976); in our studies this coincides with the LPO activities on Days 2–3. Since the oestrous cycle of the guinea-pig is 16 days long, the LPO reaches a maximum at the time of the next oestrous, about 18 days after delivery in our studies. The pattern is similar to that of ovarian secretion of oestrogens in guinea-pigs, the increase in ovarian venous plasma oestradiol-17β also being about 5-fold (Joshi, Watson and Labsetwar, 1973). Our findings are also consistent with the extensive alveolar development oestrogen causes in the guinea-pig mammary gland (Folley, 1952). Kern, Wildbrett and Kiermeier (1963) have also shown an increase in peroxidase activity in bovine milk which corresponds with ovulation. The apparent maintenance of a high LPO activity after Days 20–22 may be due to the persistence of oestrogen effects and possibly the ending of lactation. From the above evidence it would appear that lactoperoxidase in the mammary

glands of the guinea-pig may be added to the growing list of oestrogen-responsive peroxidases (Cockle and Harkness, 1978).

This rise in LPO activity by the third week *post partum* coincides with a rise in the bactericidal properties of the milk against *Esch. coli* which was not detected previously (Stephens and Dolby, 1978) as sows had not been milked beyond the 2nd week of lactation. This bactericidal activity can be reversed by agents which interfere with the antibacterial action of lactoperoxidase but not by iron, which reverses the lactoferrin/antibody system. The correlation between LPO and bactericidal activity demonstrated in Figs. 1 and 3 is not entirely consistent, however. Occasionally the antibacterial activity of the milk increases a few days before the increase in LPO activity and the reason for this is not certain. Since the activity is reversed by penicillamine or cysteine it may be that one of the other components of the LPO system has increased, as these were not monitored in the tests.

Reiter *et al.* (1976) have shown levels of LPO in bovine milk sufficient for bactericidal activity, and it would therefore be expected that guinea-pig milk collected during the first week after parturition, which has LPO activity at least 3 times higher than the average activity found in bovine milk, would also show bactericidal activity. Reiter stressed, however, the need for adequate levels of SCN^- and H_2O_2 for the LPO system to be effective against *Esch. coli*. Thiocyanate has been shown to occur naturally in many secretions including bovine milk (Reiter *et al.*, 1976) and human milk (Gothefors and Marklund, 1975) and the latter authors also demonstrated thiocyanate in infants' saliva. Hydrogen peroxide is not known to be present in sterile milk and must therefore be produced exogenously. Hamon and Klebanoff (1973) used *Streptococcus mitis* as a source of H_2O_2 in the LPO system, and suggested microbial metabolism as a source *in vivo*. Several of the guinea-pig milks tested were contaminated with *Strep. faecalis*, but heating of these milks to 63° for 30 min did not diminish their bactericidal (or LPO) activity. Addition of viable *Strep. faecalis* organisms (isolated from guinea-pig milk) to the bactericidal test did, however, increase the activity of previously inactive milks (Stephens, unpublished results). Gothefors and Marklund (1975) analysed LPO activity in human and bovine milk and saliva, and showed low activity in human milk (about 5%) when compared with bovine milk, but demonstrated LPO and thiocyanate in infants' saliva and bovine milk sufficient to inhibit bacterial growth *in vitro*. They suggested bacteria as a possible source of H_2O_2 for the system to be active *in vivo*. Since the guinea-pig gut is normally colonized with catalase-negative organisms such as *Lactobacilli* (Smith and Crabb, 1961) it would seem this system could provide antibacterial activity *in vivo* in the newborn guinea-pig, as these organisms would provide the H_2O_2 which was absent in our *in-vitro* tests.

Oral infection of the suckling young with *Esch. coli* during the first week *post partum* did not alter the quantity of lactoperoxidase found in the milk throughout lactation; however, there was a change in the bactericidal/bacteriostatic activity. Selection of guinea-pigs for this group was random, but unfortunately more had milk with bacteriostatic activity against the milk-sensitive strain before infection than in the normal group. This activity was always reversed by iron and was attributed to lactoferrin. Any initial activity did not interfere with the increase in bactericidal activity found in sows with normal young and did not appear to affect the response in sows suckling infected young. In the infected group bactericidal milks were found less frequently than in the normal group as the LPO activity increased, but bacteriostatic activity against both strains occurring later in lactation was reversed by iron, or by iron plus penicillamine (or cysteine) but not by penicillamine alone, suggesting an increase in specific antibodies and/or lactoferrin in response to infection. The reason that this increase

interfered with the bactericidal action of the LPO-Scn^--H_2O_2 system is not fully understood, but again it may be due to insufficient quantities of the components necessary for the system to be active *in vitro*. Direct measurement of the specific antibody and lactoferrin levels is desirable, as indirect measurement by reversal of the bacteriostatic activity is complicated by the presence in the milk of other antibacterial systems. Unfortunately the very small quantities of guinea-pig milk obtainable make raising of specific antisera impractical.

Appearance of bacteriostatic activity due to antibody and lactoferrin in the mothers' milk following oral infection of the young suggests that this system is being stimulated by the infection. The relative importance of this system in protecting against infection *in vivo* in the presence of so much LPO is not known, but the anaerobic conditions in the small intestine may inhibit the LPO activity, and the lactoferrin/antibody system may then be more effective.

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